Adolescent social stress does not necessarily lead to a compromised adaptive capacity during adulthood

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ADOLESCENT SOCIAL STRESS DOES NOT NECESSARILY LEAD TO A COMPROMISED ADAPTIVE CAPACITY DURING ADULTHOOD: A STUDY ON THE CONSEQUENCES OF SOCIAL STRESS IN RATS

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Abstract—Childhood bullying or social stress in adolescent humans is generally considered to increase the risk of developing behavioral disorders like depression in adulthood. Juveniles are hypothesized to be particularly sensitive to stressors in their environment due to the relatively late maturation of brain areas that are targeted by stress such as the prefrontal cortex and hippocampus. In our study male adolescent rats were subjected to repeated social defeat on postnatal day (PND) 28, 31 and 34 (experiment 1) or to daily social defeats between PND 35 and 42 (experiment 2). Adolescent rats in experiment 1 were socially housed in pairs with a male of similar age during and after the social defeat. In experiment 2 adolescents were housed either alone or with an age-mate for 7 days (PND 35–42) next to either a highly aggressive or a non-aggressive adult male neighbor with whom a repeated physical interaction was allowed. In experiment 1 the adolescent defeats affected subsequent play behavior with the cage mate. Socially stressed rats more frequently initiated play behavior but also adopted more frequently submissive postures during the play fights. As adults, they seemed to cope behaviorally and physiologically better with a similar exposure to a residential aggressive male rat than unstressed controls. In experiment 2 acute effects of adolescent social stress were studied on neuroplasticity markers like hippocampal cell proliferation and neurogenesis as well as hippocampal brain-derived neurotrophic factor (BDNF) levels. The 2nd experiment also studied long-term effects of the adolescent stress in the response to an adult social defeat. A few acute but minor changes in brain plasticity markers and behavior were observed but these were transient and no behavioral or physiological effects persisted into adulthood. The results from both experiments support the theory developed in the so-called ''match–mismatch hypothesis'' which claims that the final consequence of childhood adversity depends on how well the early life environment matches the challenges in later life. Socially stressed adolescents are rather resilient to the lasting behavioral and physiological effects of the stress exposure if they are socially housed afterward and have the ability to recover.

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Key words: adolescence, social defeat, social housing, play behavior, neurogenesis, match–mismatch hypothesis.

INTRODUCTION

Many animal species live in complex social structures. The interaction with conspecifics constitutes a pivotal part of their total environment as each individual depends heavily on its social environment for the maintenance of health, fitness and survival (Ren et al., 1999; Neumann, 2009; Dunbar, 2010; Weidt et al., 2012). However, the social environment not only has positive effects on physical and mental well-being. In case social interactions become unpleasant or even threatening, individuals experience social stress which may produce stress pathology in animal models reflecting for instance an increased risk for depressive disorders in humans (Brown and Prudo, 1981; Post, 1992; Cutrona et al., 2005; Kessler et al., 2010). A seriously compromised social environment is experienced by a substantial amount of children being victims of bullying peers (Juvonen et al., 2003). Some of the victims of bullying are reported to suffer from loneliness, low self-esteem, social withdrawal and have an increased risk to develop mood disorders like anxiety or depression (Bjorkqvist, 2001; Hemphill et al., 2012). Positive social interactions, however, such as social support from friends and family during a period of stress improves the chance of successfully coping with stress or recovering from psychopathologies as mentioned above (Franks et al., 1992; Weihs et al., 2005; Cosley et al., 2010; Dunbar, 2010; Weidt et al., 2012).

Because the social nature of our environment greatly influences our health and general well-being it is important to use appropriate animal models to uncover the underlying mechanisms by which our social environment may either positively or negatively affect our well-being. In male laboratory rodents the best equivalent of bullying is social defeat in the resident/intruder test (Koolhaas et al., 1997). In the resident/

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromodeoxiuridine; CORT, corticosterone; DCX, doublecortin; PBS, phosphate-buffered saline; PND, postnatal day; SD, social defeat; sem, standard error of the mean; WTG, Wildtype Groningen rats.

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intruder test, the intruder is repeatedly subjected to attacks and threats from the dominant resident, and these attacks often persist even after the intruder signals defeat by displaying behavioral submission, similar to victims of bullying who are harassed and assaulted even though they do their utmost to avoid provoking the bully (Bjorkqvist, 2001; Vidal et al., 2007; Watt et al., 2009). The social defeat paradigm has been used successfully in adult laboratory rodents to elucidate some of the relevant neurobiological, physiological and behavioral changes caused by acute or chronic social defeat experience (Tornatzky and Miczek, 1993; Meerlo et al., 1996; Buwalda et al., 1999). In adult males, these effects have been demonstrated to persist long after the original defeat experience when the experimental intruder animals are singly housed following the defeat (Meerlo et al., 1996; Buwalda et al., 1999). As for the beneficial aspect of the social environment such as social support during stress, adult laboratory rodents have been shown to respond positively to the presence of a non-hostile companion much like humans respond to the presence and support from a good friend during hardship (Ruis et al., 1999; Wilson, 2001; de Jong et al., 2005; Nakayasu and Ishii, 2008; Hennessy et al., 2009; Cherng et al., 2010; Macone et al., 2011). Hence, different social housing conditions during stress may serve as a useful rodent model for studying the buffering effect of social support.

Most research on social defeat stress and social support is performed using adult laboratory rodents. Yet, human peer victimization or bullying behavior is highest in juveniles (Frisen et al., 2007). Several studies suggest that juveniles and adolescents respond differently to stress when compared with adults. Therefore, they may also be affected differently in the long term (Avital and Richter-Levin, 2005; Romeo, 2010; Bingham et al., 2011). Indeed, in response to acute restraint stress, the plasma concentrations of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) remain elevated significantly longer in 28-day-old juvenile rats than in adults (Romeo, 2010). After repeated exposure to restraint stress the initial CORT response to restraint also remains higher in adolescents (Romeo, 2010; Bingham et al., 2011), but the recovery is faster than in adults (Romeo, 2010). When exposed to social defeat, single-housed adolescent rats (postnatal day [PND] 36) spent more time burying in the defensive burying test compared with undefeated controls, indicating a more proactive coping style, whereas the opposite effect was observed in adult defeated rats. This increase in burying behavior seems to be typical for social stress during early adolescence since the effect disappears in late adolescence (PND 51) (Bingham et al., 2011).

A possible explanation why brain and behavior of juveniles and adolescents may be affected by stress in a different or stronger way than adults is the relatively high developmental plasticity in brain areas that are targeted by stress such as the prefrontal cortex and hippocampus (Chechik et al., 1999; Leussis and Andersen, 2008; Stranahan, 2010). Compared to adults, juveniles and adolescents are suggested to be more susceptible to input from their environment as an essential part of learning about and adapting to their environment (Belsky et al., 2009; McCormick, 2010; Romeo, 2010; Jankord et al., 2011; Schmidt, 2011). Consequently, they may be particularly vulnerable to the detrimental effects of stress and adverse environment.

Not all children exposed to an adverse social environment develop psychopathologies as adults and individuals who experience a relatively trouble-free childhood may still develop mood disorders (Ellis et al., 2011; Schmidt, 2011). Even when considering genetic predisposition to stress, the connection between childhood adversity and adult mental health is complex. Since there is a clear learning component in experiencing stressful situations, it is also possible that previous confrontations with stressors lead to an enhanced ability to successfully cope with later stress situations of a similar nature. This idea is summarized in the match–mismatch hypothesis that suggests that the final consequence of childhood adversity depends on how well the early life environment matches the challenges in later life (Ellis et al., 2011; Schmidt, 2011; Daskalakis et al., 2012).

In the current study it is tested in rats whether social defeat stress during adolescence results not only in acute changes in brain and behavior but also longer lasting changes in behavior indicative of an increased susceptibility to stressors of either similar or different nature. As mentioned above, it is known that social defeat stress in adult rodents produces long-lasting effects on brain, physiology and behavior when these animals are singly housed after the defeat and that social housing ameliorates the negative consequences of the social stress (Ruis et al., 1999; de Jong et al., 2005). During development it is even more important that animals are allowed to have social interactions with their peers. Play deprivation or deprivation of social contacts can, depending on the timing, lastingly affect the development of normal social behavior which coincides with alterations in neurochemistry and neuroplasticity. Social play during adolescence, with play fighting being the most commonly expressed form, is crucial for the development of adult social competence (Einon and Morgan, 1977; van den Berg et al., 1999; Pellis and Pellis, 2007). There are critical periods for the effects of social isolation in rats. Einon and Morgan (1977) showed that depriving rats of social interactions between PND 25 and 45 leads to irreversible decreases in voluntary exploration of novel areas and objects whereas deprivation between PND 16 and 25 or after PND 45 did not lead to irreversibility in the development of these behaviors. The lasting effects of social isolation during postnatal weeks 4 and 5 on adult social behavior could be normalized by social re-housing of adolescents after the isolation period (Hol et al., 1999). For this reason we did not socially isolate our experimental adolescent animals during this crucial phase after the social stress experience. Furthermore, it can be questioned to what extent lasting social deprivation during this developmental period in combination with social defeat stress adds to the translational value of this animal model.

Two experiments were performed with the aim to study short- and long-lasting effects of adolescent social stress on brain and behavior. In the first experiment (experiment 1), male adolescent rats were confronted with a highly aggressive residential male on PND 28, 31 and 34. Since we hypothesized that severe social stress would affect social play behavior with an age-matched cage mate, we observed play behavior until adulthood. Furthermore, we studied long-term effects on anxiety and social behavior as well as the behavioral and physiological response to adult social defeat. The physiological stress response was measured using permanently implanted radiotelemetry transmitters. In a second experiment (experiment 2), the effects of more chronic social stress were studied. Male adolescent rats were housed in close contact with either a highly aggressive or a non-aggressive adult male conspecific for 1 week (PND 35–42) (Kudryavtseva et al., 1991). During these 7 days a transparent separator wall was withdrawn 10 times for 10 min allowing physical contact between the adolescent experimental animals and the adult male. Aggressive adults repeatedly attacked the experimental rats during these 10-min periods of physical contact. To study the possible protective role of social housing during this social stress period, experimental rats were housed either alone or with an age-matched male cage mate in the compartment next to the adult neighbor. Shortly after the last interaction, neuroplasticity was studied in brains of stressed and non-stressed animals in one group of animals. In a second group of animals in experiment 2 long-term effects were studied on anxiety and social behavior and on behavioral and physiological responses to adult defeat.

EXPERIMENTAL PROCEDURES

Experiment 1

Animals. Male Wistar rats ($n = 32$) were obtained from the Harlan Laboratories (Horst, The Netherlands) at PND 21–23. Upon their arrival, the animals were housed in pairs in translucent plastic cages ($40 \times 25 \times 15$ cm). Food and water were supplied *ad libitum* with temperatures of approximately 21°C under a 12:12 h day–night regime (lights on at 20.00 to secure that all manipulations and tests were performed during the active phase (subjective night) of the animals. All animals were given 1 week to habituate to the light regime, food, handling and presence of the experimenter in the room before the first experimental manipulation. Animals were randomly assigned to receive social defeat, control procedure or a neutral cage mate. On PND 25, animals assigned to be housing partners were marked with black hair dye on their back to allow a quick discrimination between the un-manipulated and the control/defeat animal during behavioral observations. After the last stress/control treatment on PND 34, the pairs were transferred into a bigger translucent plastic cage ($55 \times 34 \times 18$ cm) until postnatal day 70 to give them enough space to show unrestrained social and play behavior. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC-RuG) of the University of Groningen.

Adolescent manipulations. All manipulations and tests took place between 08.00 and 12.00 h (see timeline in Fig. 1).

Social defeat stress. Social stress was inflicted by repeated social defeats by highly aggressive adult male Wildtype Groningen rats (WTG). The male aggressive WTG's were selected on their high offensive aggression toward an intruder in the resident–intruder paradigm (de Boer et al., 2003). Resident rats were at least 6 months of age. They were housed in a separate room in large cages ($80 \times 55 \times 40$ cm) with an ovary-ligated female to stimulate territorial aggression. The residents were trained on a regular basis by confronting them with naïve male adult intruders. Before the start of the experiments, residents with attack latencies shorter than 2 min were selected. By using residents with more or less equal readiness to attack, we were able to reduce variation in conflict intensity to a minimum. Thirty minutes prior to the interaction with an experimental intruder male, the females were removed from the residents' cage. On PND 28, 31 and 34 the experimental animals were transferred in an unfamiliar cage to the room of the residents where they were immediately placed into the territory of the resident. The resident rat started inspecting the unknown intruder showing threatening postures that rapidly changed into physical attacks (lunging, kicking, and biting). This motivated the experimental adolescent rat to show submissive behavior (i.e. lying on the back, stiffly putting the paws upward) which frequently persisted even when the resident turned away. The interaction was continued until the experimental animal received three serious defeats characterized by a subsequent submissive posture with a maximal interaction time of 10 min. Subsequently, the Wistar rats were transferred into a wire mesh protection cage ($30 \times 14 \times 14$ cm) that allowed visual, auditory and olfactory contact to the resident but prevented direct physical attacks. The wire mesh protection cage remained in the resident cage until a total time of 60 min had passed. The experimental animals were removed from the wire mesh protection cage and transferred to their room. There they remained in their transport cages (equipped with food and water) for 5 h before they were placed in their own home cage again. This time of isolation from their housing partner was included to prevent the effect of the stress treatment to be washed out immediately by social contact. At PND 31 the social stress only comprised psychological threat, i.e. 60 min visual, auditory and olfactory contact to the wildtype resident while being in the wire mesh protection cage. The social stress at PND 34 was similar to that on PND 28 (rats were physically attacked and defeated).

The social defeat procedure for rats in this phase of early adolescence was new for us, and it was uncertain if resident male WTG rats would recognize animals this young as ''attack-worthy'' subjects. It turned out, that the fight-trained WTG rats did attack the adolescent males of approximately one fourth their own size readily and ferociously. All adolescent animals received at least three attacks upon which they assumed a clear submissive posture.

Control treatment. Alternatively to the stress treatment, the animals of the control group were transported to a room adjacent to the residents' room, which was similarly lit. They were put into a novel, unknown cage, which resembled a WGT resident's cage in proportions. After 10 min, the control animals were put into a wire mesh protection cage ($30 \times 14 \times 14$ cm) where they remained for further 50 min. After these 60 min the animals were removed from the wire mesh protection cage and retransferred to their room, where they remained in their transport cages (equipped with food and water) for 5 h until they were placed in their own home cage again. Together with the animals of the defeat group they received a control treatment at PND 28, 31 and 34.

Observation of play behavior. All behavioral observations were performed by one experimenter in dim red light between 09.00 and 15.00 h during the active phase of the animals. In

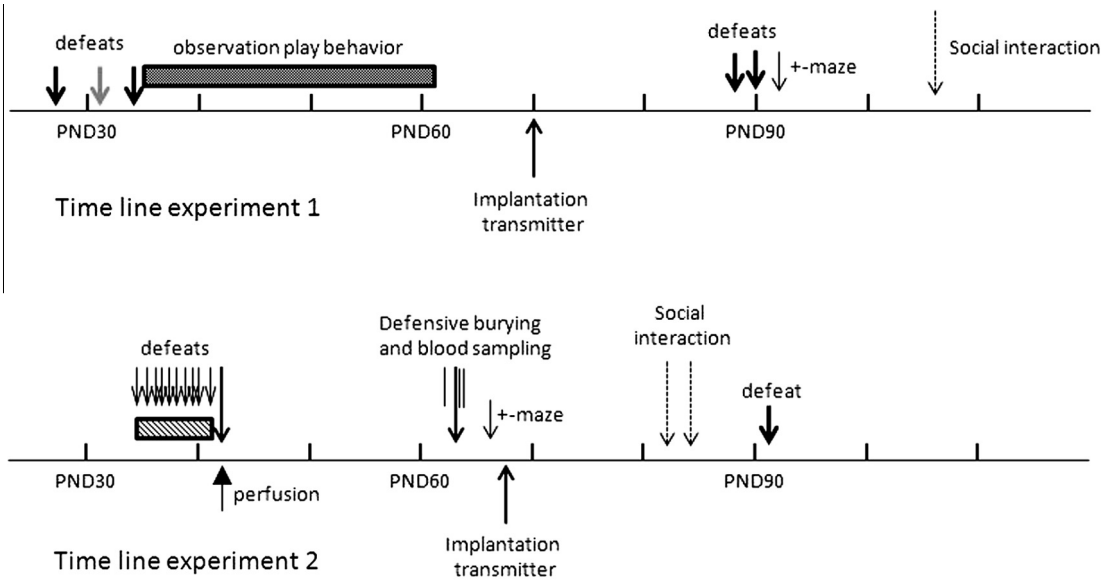

Fig. 1. Graphical illustration of the time line of experiments 1 and 2, displaying the temporal location of treatment and tests on the short- as well as the long-term effects. Ticks indicate intervals of 10 days. Defeats are indicated by bold black arrows; in experiment 1 the psychological stress by exposure in the wire mesh cage to the residential male is indicated by a bold gray arrow.

Table 1. Definition of the behavioral categories that were scored in experiment 1 during 10 min of observation

Behavior	Definition
Initiating play	Approaching the partner in a playful manner, i.e. jumping at, running from, slight boxing/wrestling, nipping body parts
Undergoing play	Wrestling, running/jumping/chasing, boxing, kicking in a reciprocal manner; nipping, jumping on, boxing while partner defends himself or vice versa
Submissive posture	Lying (motionless) on the back, throat exposed to partner, no defense in form of boxing or kicking
Dominant posture	Lock partner in submissive pose (with forced grooming/licking), tower over the partner (pinning)
Approaching	Neutral movement toward the partner
Avoiding	Moving away from the partner (walk, run), rotating body axis away from the partner
Jump and run	Intense locomotion without obvious target
Eat and drink	Consuming food pellets or drinking from the water bottle
Social exploration	Sniffing and/or touching the partner (physical contact within whisker-length)
Cage exploration	Sniffing at, moving around in the cage (bedding, gnawing stick, bars), climbing, digging, gnawing
Sleep/rest double	Relaxed position, sleeping with partner in physical contact (whisker-length)
Sleep/rest single	Relaxed position, sleeping without physical contact to partner
Grooming self	Licking, gnawing, combing own body
Grooming other	Licking, gnawing, combing body of the partner
Being groomed	Being licked, gnawed at, combed by partner
Attack	Lunging at, biting the partner
Chase	Run after the avoiding/fleeing partner
Flee	Being chased by and/or running away from the partner

total 14 observations were performed (on average three times a week in irregular intervals), beginning on PND 35 and ending on PND 62. Each animal from the defeat and the control group was observed for 10 min in its home cage where it could freely interact with its cage mate. Every 10 s, the behavior of the experimental animal (i.e. from defeat or control group) at exactly this time point was recorded and designated to one of 18 behavioral categories described in Table 1.

Adult manipulations. Surgical implantation of the telemetry sensor. For the surgical implantation of telemetry sensors (TA-40, Data Sciences International, St. Paul, MN, USA) around PND 70, all rats were anesthetized with a mixture of isoflurane and oxygen. After surgery, the animals were placed in a smaller cage type ($40 \times 25 \times 15$ cm), in which they were single-housed until the end of the experiment.

Telemetric recording. Measurements of body temperature and home cage activity by the implanted sensors were recorded with sensor plates and processed by the LabPro computer program (Data Sciences International, St. Paul, MN, USA). All measurements were taken in 5-min intervals, starting the first day after surgery (PND 71) and recording until the end of the experiment. Each measuring point represents the actual body temperature of the animal at that time and an accumulative activity count of the previous 5 min.

Adult defeat (PND 89–90). The adult defeat procedure was identical to the adolescent defeat and was performed on two subsequent days. Both the adolescent defeat group and the control group were subjected to social defeat at adulthood.

Elevated plus maze (PND 91). Both treatment groups were tested for general anxiety with an elevated plus maze (height 55 cm, arm-length 45 cm) between 13.00 and 15.00. The animals were each taken to the test room and placed in the center of the maze, facing into the direction of a closed arm. Animals could move freely between two closed and two open arms and were observed by a camera (Canon, Tokyo, Japan) for 5 min. The experimenter recorded behavior in an adjacent room (by watching the camera recordings on a monitor), measuring the duration each animal spent in the open and closed arms, respectively. Full entry into an arm was considered when all four paws of the animal were placed on the surface of the respective arm. The maze was cleaned with soap and water after each rat so no olfactory distractions would influence the behavior on the maze. After the test each animal was immediately returned to its room. Performance on the maze was analyzed by calculating the relative percentage time each animal spent on the open arms of the maze (time on the open arm/[time on the open arms + time on the closed arms]).

Social interaction test (PND 106). In an open field arena (diameter, 120 cm) an unfamiliar male Wistar rat was placed in the center, encaged by a wire mesh protection cage (12 × 12 × 30 cm). Around this cage a 15 cm interaction zone was defined, which is considered to be the distance within which another rat can investigate the conspecific (e.g. vision, olfaction). Animals within the boundaries of this zone are presumably actively interested in the encaged conspecific, showing no social anxiety within this context. Experimental animals were transported to the test room and placed on a defined spot on the edge of the open field arena. Hereupon, the experimenter left the test room and the movements of the animal were recorded by the Ethovision Videotracking program (Noldus Information Technology, Wageningen, The Netherlands) for 5 min. Recordings were analyzed regarding the animal's latency to enter the interaction zone, total time spent in the interaction zone and the total distance the animal moved within the open field arena. After the test, each animal was returned to its room immediately.

Experiment 2

Animals. Male Sprague-Dawley rats were derived from the Harlan Laboratories (Horst, The Netherlands) at PND 28 and housed in groups of 2–3 siblings in standard 40 × 25 × 15 cm Plexiglas cages until the start of the experimental housing at PND 35. The rats had *ad libitum* access to food (standard chow) and water, except during the 10-min. interactions with the adult, during which the water bottles were removed. Animals were housed in a climate-controlled room (21 °C) under a 12–12-h light/dark cycle (lights on at 22:00 h). All tests were performed during the dark-phase under dimmed lighting conditions when possible. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC-RuG) of the University of Groningen.

Adolescent manipulations. Housing conditions. At PND 35 the experimental adolescents were housed either alone or with a non-sibling age-matched cage mate (partner) in one half of a large home cage (80 × 55 × 40 cm). The two halves of the home cage were separated by a transparent Plexiglas separator. The other half of the cage was occupied either by an adult aggressive WTG male rat together with an ovary-ligated female (which stimulates territorial aggressive behavior) or an adult non-aggressive Wistar male. The male aggressive WTGs were selected on the basis of their high offensive aggression toward an intruder in the resident–intruder paradigm (de Boer et al., 2003). The cages with experimental animals housed next to a WTG male were in a different room than the cages where experimental rats were housed next to a non-aggressive Wistar male.

Social stress procedure. During the 7 days of experimental housing (PND 35–41) experimental animals were exposed to 10 interactions (each lasting 10 min) randomly distributed over the dark phase of the day with their adult male neighbor by removing the separator, giving the adult male and the experimental adolescent unrestricted access to the whole cage. The adult female partners were removed 30 min before the interaction and the adolescent partner animals were removed immediately before the interaction (see timeline in Fig. 1). During these interactions, the adolescents housed with a WTG neighbor were repeatedly fiercely attacked and defeated whereas the rats housed with a Wistar neighbor were not, thus creating 4 different housing groups; singly housed defeated adolescents (SD; $n = 15$), pair-housed defeated adolescents (PD; $n = 16$), singly housed non-defeated control adolescents (SC; $n = 15$) and pair-housed non-defeated control adolescents (PC; $n = 13$).

Interaction 1, 2, 3, 7 and 10 were recorded on a camera and the adolescent's behavior during interaction 2 and 10 was subsequently analyzed using a custom-made analysis program (E-line). The frequency and duration of the following behavioral elements displayed by the adolescent were scored manually: Attacked/chased by the adult, assuming submissive postures, freezing, crawling under adult (but not submissive), approaching/following adult, playing with/climbing on top of adult, social exploration of adult and non-social activity (cage exploration and grooming). During interaction 4, 5, 6, 8 and 9 the predominant behavior of the adolescent during the first and last 20 s. of the interaction was recorded in addition to attack latency and frequency of attacks by the adult and submissions by the adolescent.

Body weight. Body weight was measured every day from PND 28 to 42 to assess the effect of treatment on body weight gain. After the experimental housing the animals in the long term study were weighed 2–3 times a week.

Defensive burying. After the last interaction on PND 41 the adolescents were either left in their own half of the home cage and sacrificed and perfused the next day (short term study; SD ($n = 7$); PD ($n = 8$); SC ($n = 7$) and PC ($n = 5$)) or placed into a defensive burying cage (24 × 24 × 30 cm) along with their partner (if pair housed) (long term study; SD ($n = 8$); PD ($n = 8$); SC ($n = 8$) and PC ($n = 8$)). The defensive burying cage was placed inside the resident/intruder cage without the transparent separator to provide continuous audio–visual and olfactory exposure to the neighboring male adult until the first defensive burying test was performed on PND 42. At this day each defensive burying cage was carried to a secondary testing room immediately before testing. The experimental adolescent was briefly removed while the shock-prod was placed approximately 2 cm above the bedding, after which the test was performed (de Boer and Koolhaas, 2003). Upon touching the electrified prod adolescents received an electric shock of 2 mA and 2000 V. A full behavioral profile was recorded for 10 min after first electric shock. In addition latency to bury was recorded.

After the defensive burying test the adolescents in the long-term study were socially housed with similarly treated age mates in groups of 2–4 adolescents per cage. The adolescents remained in these housing groups until PND 88, after which they were solitary housed (for complete timeline see Fig. 2).

Immunohistochemical staining and quantification of hippocampal cell proliferation, neurogenesis, and BDNF. Approximately 24 h after the last interaction with an adult male, the adolescents in the short-term study were injected i.p. on PND 42 with (20 mg/ml saline) 5-bromodeoxiuridine (BrdU) solution (300 mg/kg body weight). Two hours after injection with BrdU the adolescents were deeply anaesthetized with an overdose of sodium pentobarbital (i.p.) and perfused with heparinized saline (10 ml [4000 IU] heparin/1 L saline) for 1 min., followed by 4% (wt/vol) paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 10 min. The brains, thymus and adrenal glands were removed, and the brains were stored in 0.1 M PBS at 4°C and subsequently cryoprotected in 30% (wt/vol) sucrose solution in 0.1 M PBS for 24–28 h and stored at −80°C until cryostat sectioning (40 μm).

BrdU staining. Coronal cryostat sections (40 μm) were made of the entire hippocampal structure. For the BrdU staining every 12th hippocampal section was collected. Sections were incubated overnight with biotinylated Rat-anti BrdU antibody (1:800, Serotec: mca2060) at 4°C, and then incubated with biotinylated donkey-anti-rat immunoglobulin (1:400, Jackson 712065153) for 45 min at room temperature. The number of proliferating cells in six subsequent rostral sections was quantified in the dorsal dentate gyrus (between bregma −2.0 mm and bregma −4.5 mm (Paxinos and Watson, 2008). BrdU-positive cells were counted in the subgranular zone of the dentate gyrus (defined as a two-cell body-wide zone on either side of the border of the granular cell layer and the hilus) throughout the entire thickness of each hippocampal section. Total estimation of BrdU-positive cells in the dorso-rostral hippocampus was made by multiplying the sum of the counts by 12.

Doublecortin (DCX) staining. Sections were incubated for 3 days (72 h) in rabbit-anti-DCX (1:1000, Cell Signaling Technology, Inc.) at 4°C, and then incubated with biotinylated rabbit-anti-goat immunoglobulin (1:500, Jackson ImmunoResearch Laboratories, Inc.) for 2 h at room temperature. Three similar sections from each animal were analyzed for DCX active cells in the dorsal dentate gyrus (between bregma −3.3 mm and bregma −4.0 mm (Paxinos and Watson, 2008).

BDNF staining. Sections were incubated for 3 days (72 h) in rabbit-anti-BDNF (1:250, Alamone Labs) at 4°C, and then incubated with biotinylated rabbit-anti-goat immunoglobulin (1:500, Alomone Labs Ltd.) for 2 h at room temperature. Three similar sections from each animal were analyzed for BDNF active cells in the dorsal hippocampus and dentate gyrus (between bregma −3.3 mm and bregma −4.0 mm (Paxinos and Watson, 2008).

Sections from all stainings were mounted on glass slides, dehydrated by means of alcohol–xylol treatment and embedded in DPX-mounting media. DCX and BDNF sections were analyzed using an optical microscope and % area covered with immunolabeling and optical density of immunopositive staining was quantified using a Quantimet 550 image analysis system (Leica).

Adult manipulations. Defensive burying. At PND 63 the defensive burying was repeated. In this second test all experimental animals were housed alone in the defensive burying cage from PND 63 to 64 and the shock–prod was not turned on.

Corticosterone (CORT) response. Baseline blood samples were drawn by tail clipping at PND 62, 4–5 h into the dark phase the day before the animals were placed into the defensive burying cages. At PND 63 the first sample was drawn immediately after the defensive burying test (12–15 min after the start of the defensive burying test) and the second sample was drawn 60 min after the start of the defensive burying test. Heparinized blood samples were kept on ice until centrifuging and collected plasma was stored at −20°C until analysis to determine CORT concentrations using ImmuChem™ Double Antibody Corticosterone ^{125}I RIA kit (cat. No. 07-120102; MP Biomedicals, LLC).

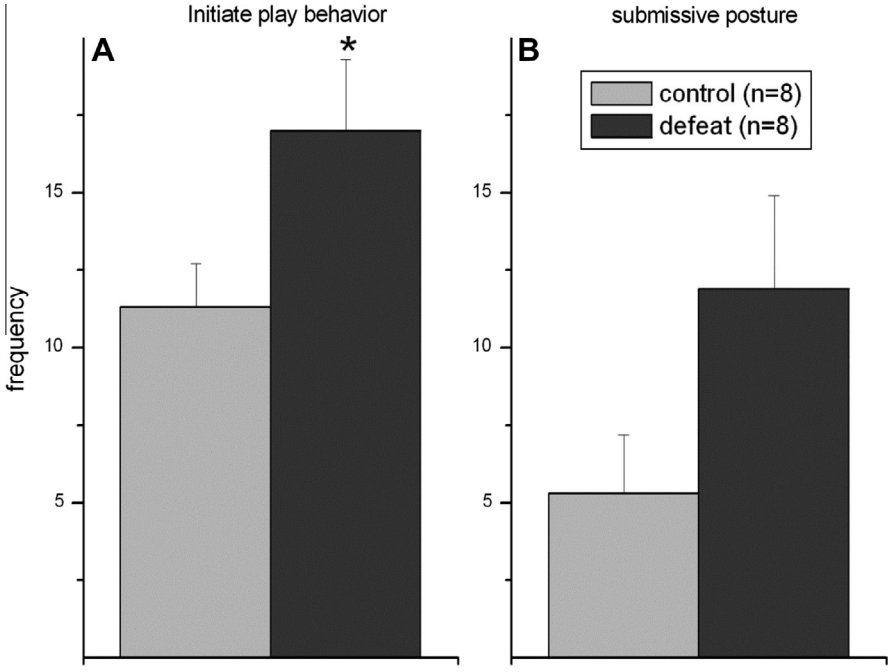

Fig. 2. Experiment 1: total number of times that animals (''control'' rats: not defeated during adolescence and ''defeat'' rats: socially defeated during adolescence) initiated play behavior and number of submissive postures during play behavior in the home cage with a male cage mate as observed during 14 observation periods of 10 min from PND 35 to 62. (means ± standard error of the mean [sem], $^{*}p < 0.05$).

Elevated plus-maze. The level of anxiety was tested in the elevated plus-maze at PND 67. Each individual was carried to a testing room immediately prior to a 5-min test. The light intensity ranged between 75 and 80 lux in the open arm to less than 1 lux in the closed arm. Number of entries into and percentage of time spent in the open and closed arms were subsequently scored using E-line.

Implantation biotelemetry transmitter and telemetry recording. At PND 68 biotelemetry transmitters (model TA10TA-F40, Data Sciences International Inc., USA) were implanted in the abdominal cavity as described under experiment 1.

Social interaction test. At PND 82 and 83 exploratory behavior was tested in an open field arena (70 × 70 cm) with a wire mesh cage (23 × 13 × 13 cm) placed along one of the sides of the arena. On the first testing day each animal was initially tested with an empty cage and one minute later with an unfamiliar adult male Sprague–Dawley rat in the wire mesh cage. On the second testing day the wire mesh cage contained an unfamiliar ovariectomized adult Sprague–Dawley female. Each testing session lasted 2.5 min. Time spent within 10 cm of the wire mesh cage and in the half of the arena most distant from the wire mesh cage was analyzed using Ethovision (Noldus Information technology, Wageningen, The Netherlands).

Adult social defeat. At PND 91 all experimental animals were again subjected to social defeat. The experimental animal was introduced as an intruder into the home cage of an unfamiliar aggressive WTG male resident for 10 min during which the experimental animal was repeatedly defeated. Attack latency was recorded and the interaction was recorded to enable later behavioral analysis.

Temperature response. Measures of baseline body temperature were obtained between PND 88 and 91. The impact of the adult social defeat at PND 91 on body temperature was measured from PND 91 to 98.

Statistical analysis of data from experiments 1 and 2. Combined effects of treatment and housing condition were analyzed by use of two-way analysis of variance (ANOVA). Subsequent analysis of treatment effects within each housing group or housing effects within each treatment group was analyzed by use of one-way ANOVA. For all tests, the software package SPSS was used (version 17.0; SPSS, Chicago, IL) Differences with p values lower than 0.05 were regarded as significant.

RESULTS

Experiment 1

Effects of social defeat stress on body weight gain and play and other behavior. Adolescent defeat did not induce differences in body weight gain between groups.

Both control and defeated rats showed vivacious play behavior and social interaction with their cage mate. Comparison of the sum of the frequency of the individual behaviors (see Table 1) showed that previously defeated adolescent rats initiated play behavior with their cage mate more frequently ($F(1,15) = 4.7$; $p < 0.05$) (Fig. 2). During these play fights they tended to show submissive behavior more often than their non-stressed cage mates ($F(1,15) = 3.6$; $p = 0.08$). No differences were observed in the other behaviors.

Aggressive behavior toward experimental animals in the adult social defeat experience. Behavioral observations during the two adult defeats indicated that the average attack latency for both defeats was significantly higher in rats that were defeated as adolescents than in controls (105 ± 25.9 s in adolescent defeat vs 44 ± 8.5 s in controls; $p < 0.05$; Fig. 3A). Moreover, also the total number of attacks received during the two defeat exposures was higher in controls than in adolescent-defeated rats (resp. 19 ± 1.9 and 13 ± 1.5; $p < 0.05$; Fig. 3B).

Circadian amplitude in day–night oscillation in body temperature following adult Defeat. The effect of adult social defeat on body temperature over days and nights before, during and after the adult defeat is shown in Fig. 4. Repeated measures ANOVA comparing day and night values from day −1 to day 5 shows that there is no significant interaction between treatment and time indicating that temperature increases in control as well as in adolescent defeated rats. However, there is a significant difference between groups in day temperature response ($F(1,14) = 9.6$; $p = 0.008$) and night temperature response ($F(1,14) = 5.3$; $p = 0.04$). Body temperature in control rats showed a stronger increase in comparison to rats that were defeated as adolescents leading to reduction of the amplitude of circadian day–night oscillation.

Anxiety behavior after adult defeat. On PND 89 both treatment groups were subjected to a social defeat, followed by a second one the next day. Rats were subsequently tested on PND 91 for anxiety using an elevated plus maze test. The mean relative percentage time spent in the open arm was similarly low for both groups with 10.8% (±15.1 standard deviation [SD]) for the defeat group and 8.6% (±10.0 SD) for the control group (data not shown).

Adult social interaction test. The social interaction 2 weeks after adult defeat did not differ between groups (data not shown).

Experiment 2

Behavioral evidence of the efficacy of the social stress procedure. Within the defeated group, six adolescents from the short-term study and one from the long-term study were removed from the analysis, because they had not been repeatedly defeated by the adult WTG neighbor. Two adolescents from the short-term study control group were not included in the analysis because they had been attacked by the adult Wistar neighbor.

The behavioral profile obtained from analysis of the 2nd and 10th interaction between the adolescents and their adult neighbor revealed significant behavioral differences between defeated and control adolescents with control adolescents showing little or no defeat-related behavior (‘‘chased/attacked’’ ($F(3,55) = 14.934$; $p = 0.000$), ‘‘submissive’’ ($F(3,55) = 10.676$; $p = 0.000$), ‘‘freezing’’ ($F(3,55) = 53.093$; $p = 0.000$)) and control adolescents

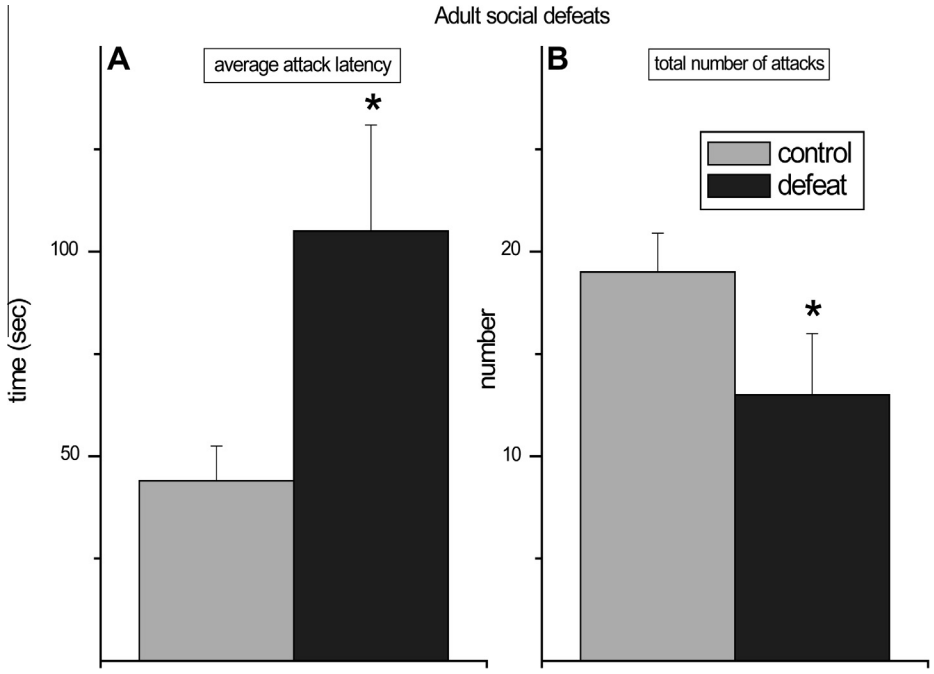

Fig. 3. Experiment1: average latency (A) and number of attacks (B) during the two adult social defeat exposures on PND 89–90 in control rats (light gray bar: ''control'' (not socially defeated during adolescence)) and ''defeat'' (black gray bar: defeated also during adolescence). (means ± sem, $^{*}p < 0.05$).

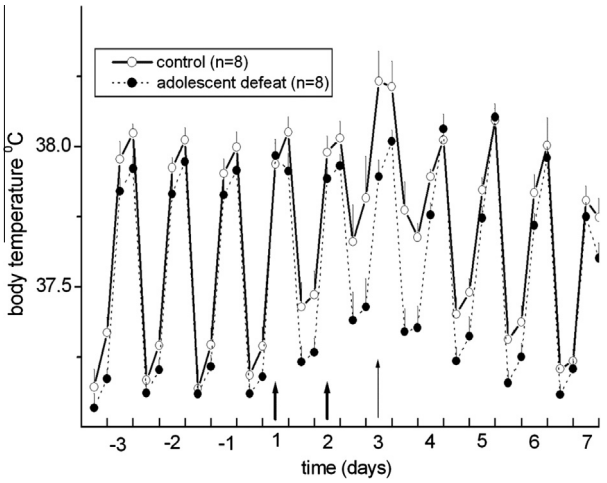

Fig. 4. Experiment 1: telemetric recordings presenting 6 h averages of core body temperature during night (the 2 peak values) and day (the 2 low values) reflecting circadian patterns in the home cage of control rats and rats defeated as adolescents 3 days before and during two subsequent adult defeats on PND 89 and 90 (indicated by fat arrows) and during 5 days following the second defeat. Exposure to the elevated plus maze on day 3 (PND 91) is indicated by a thin arrow.

performed significantly more non-defeat-related behavior than adolescents in the defeated group (''under adult'' ($F(3,55) = 40.237$; $p = 0.000$), ''approach/follow adult'' ($F(3,55) = 7.816$; $p = 0.000$), ''play/on top of adult'' ($F(3,55) = 17.167$; $p = 0.000$), ''social exploration'' ($F(3,55) = 24.976$; $p = 0.000$), ''non-social activity'' (mainly cage exploration) ($F(3,55) = 10.969$; $p = 0.000$)) (Fig. 5). There were no significant differences in behavioral response to treatment between adolescents in the short-term and the long-term study.

Within the control group, single-housed adolescents played more with the adult (($F(1,26) = 9.430$; $p = 0.005$) and spent less time performing non-social activity ($F(1,26) = 16.784$; $p = 0.000$) than pair-housed adolescents. Within the defeated group, single-housed adolescents spent significantly more time crawling under the adult ($F(1,29) = 6.544$; $p = 0.016$) compared with pair-housed adolescents (data not shown). There were no significant differences in the aggressiveness of the WTG toward single-housed compared with pair-housed adolescents (''chased/attacked'' ($F(1,29) = 0.087$; $p = 0.770$), ''submissive'' ($F(1,29) = 0.003$; $p = 0.956$), ''freezing'' ($F(1,29) = 0.218$; $p = 0.644$)). Behavioral observations from the other interactions support the findings mentioned above (data not shown).

Short-term effects of social defeat and housing. Short-term effects of social defeat treatment and housing condition during social stress was assessed on the last day of experimental housing (PND 42).

Body weight gain. Adolescents in the control group gained significantly more body weight during the experimental housing week ($F(3,55) = 9.361$; $p = 0.000$). There was a significant interaction between treatment and housing condition ($F(3,55) = 8.222$; $p = 0.006$) with control pair-housed adolescents showing the largest weight gain and defeated pair-housed adolescents showing the smallest weight gain (Fig. 6A). There was no significant difference in body weight gain between groups in the week preceding the experimental housing (between PND 28 and 35). Within

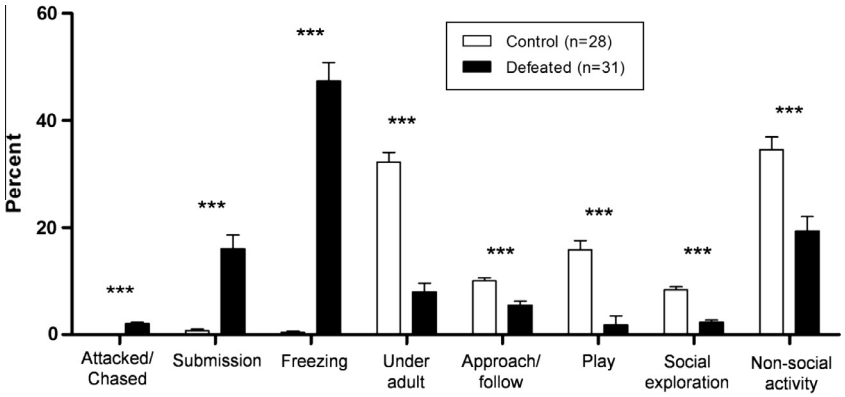

Fig. 5. Experiment 2: full profile of the behavioral elements performed by the experimental adolescents during a 10-min interaction with either an adult, non-aggressive, Wistar male (''control'') or a highly aggressive Wildtype Groningen male (''defeat'') irrespective of housing condition, measured as the mean (±sem) percentage of time spent in each behavior during interaction 2 and 10 (PND 36 and 41). Defeated adolescents spent more time performing defeat-related behavior compared with control adolescents, whereas control adolescents spent more time performing non-defeat-related behavior. Significant differences between control and adolescent defeated rats is indicated by asterisks; $^{***}p < 0.001$.

the pair-housed group there was no significant difference in body weight gain during housing between the experimental adolescents and the partner animals.

Adrenal and thymus weight. There was no significant effect of treatment and housing conditions on total adrenal and thymus weight at PND 42 (data not shown).

Cell proliferation – BrdU. Two animals were excluded from the analysis because the correct injection could not be verified. Control pair-housed adolescents had a significantly higher number of BrdU-positive cells in the dorsal hippocampus compared with defeated pair-housed adolescents ($F(3,19) = 3.251$); $p = 0.045$) (Fig. 6B).

Neurogenesis – DCX. Two animals were excluded from the analysis because the quality of the sections was poor. Defeated pair-housed adolescents had a significantly smaller area covered with DCX immunostaining in the molecular layer of the inner blade of the dentate gyrus compared with pair-housed control adolescents ($F(3,25) = 1.750$; $p = 0.046$) (Fig. 6C). No significant effect of treatment or housing was seen in the inner blade of the dentate gyrus (data not shown).

BDNF immunocytochemistry. Although optical density of BDNF immunocytochemistry in the granular cell layer of the outer blade of the dentate gyrus was somewhat reduced in defeated adolescents this difference did not reach the level of significance ($F(1,27) = 1.349$; $p = 0.066$) (data not shown).

Defensive burying test. On PND 42 the defensive burying in defeated adolescents was not significantly different from that in control-treated rats.

Long term effects of social defeat and housing. Long-term effects of repeated adolescent social defeat and housing condition during social stress was assessed between PND 63 and 120. No differences were found between groups in any of the measured physiological (CORT response and temperature measurements) and behavioral parameters (elevated plus maze and social interaction behavior) (data not shown).

DISCUSSION

The main conclusion from this study is that the Wistar rats in experiment 1 and the Sprague–Dawley rats we used in experiment 2 are resilient to lasting negative effects of adolescent social stress. The social housing after the adolescent social stress experience may play an important role in this. In adult defeat studies it has been shown that social housing ameliorates the effects of the social stress (Ruis et al., 1999). Lasting social deprivation during this crucial period for the development of normal social behavior, however, results in the development of abnormal social capacities (Hol et al., 1999; van den Berg et al., 1999) and since it was not our primary aim to study the effects of social deprivation, lasting social deprivation would hamper the interpretation of the findings after social stress in the form of social defeat. Social isolation possibly also interferes with the translational value of the model. If one considers bullying as the human equivalent of social defeat in animal models, it is important to realize that also bullied children are having social contacts during and after the bullying phase. Quality of the social contacts available to bullied children may play a role, however, in the development of behavioral disorders in later life. Despite the social housing, we did not anticipate seeing so little effect of this intense social stressor. The WTG residents we used were extremely aggressive and the conflicts in that sense can be regarded as potentially life-threatening situations. We expected that, despite the social housing, this intense stress during adolescence would leave behavioral and neurobiological traces such as lasting changes in the brain and behavior indicative of a decreased well-being.

In experiment 1 where adolescent rats were exposed to repeated defeats in the resident–intruder paradigm, the

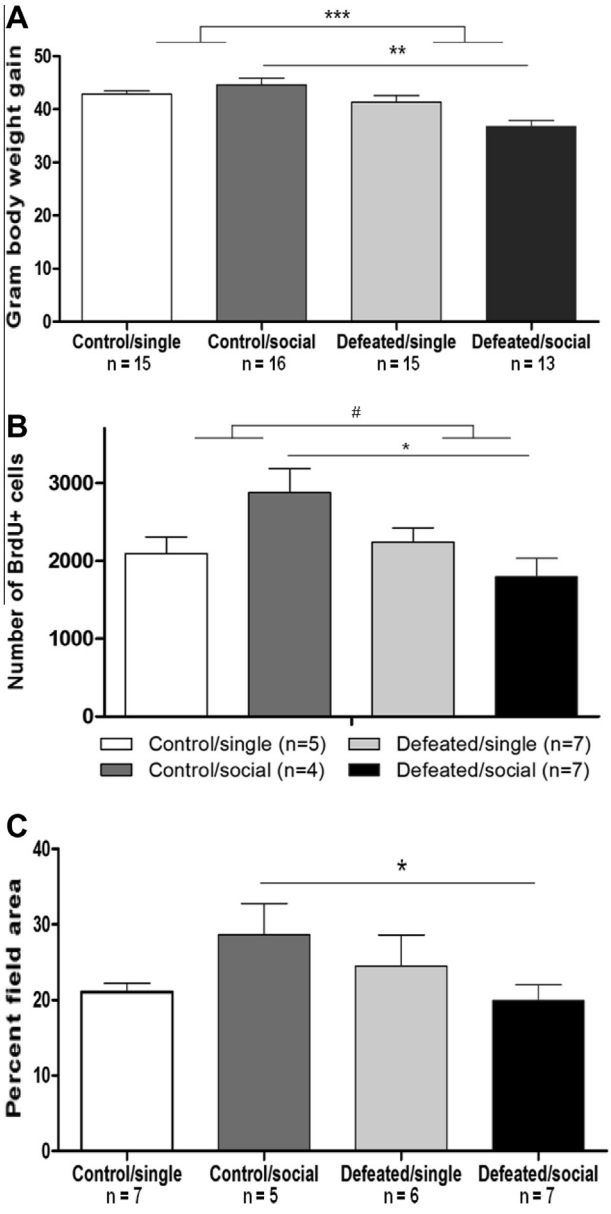

Fig. 6. Effect of treatment and housing condition on: (A) body weight gain during the experimental housing week from PND 35 to 42. Control adolescents gained more weight than defeated adolescents and a significant interaction effect was observed between treatment and housing producing the lowest overall weight gain in defeated pair-housed adolescents; (B) the number of BrdU-positive cells in the dentate gyrus of hippocampus 2 h after injection with BrdU at PND 42. A significant interaction effect was observed between treatment and housing with the lowest mean number of BrdU-positive in the defeated pair-housed group; (C) field area covered by DCX immunostaining in the inner blade of the dentate gyrus at PND 42, with defeated adolescents having a significantly lower number of DCX-positive cells. Mean values (±sem); $^{\#}p = 0.066$ (5B); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

defeat affected subsequent play behavior with the cage mate. Of all behavioral categories scored only play initiation was more frequently observed in the socially stressed rats as well as a more frequent occurrence of submissive postures during these play fights. Play behavior is rewarding for young rats (Burgdorf et al., 2008) and the increased initiation of play while showing more submission supports the findings of Pellis and Pellis (1992) in which they found subordinate male rats to initiate more play fights and show more submission than their dominant cage mates, both in adolescence and in adulthood. They described this to function as "friendship maintenance mechanism permitting co-existence in multimale colonies" (Pellis and Pellis, 1992).

A striking and surprising finding in experiment 1 was that the circadian body temperature following an adult social defeat was less affected in the rats that were defeated as adolescents than rats that did not experience this adolescent stress. The typical increase in body temperature, especially during the resting phase of the day, resulting in a flattening of the circadian amplitude following defeat (Meerlo et al., 1996; Buwalda et al., 2001) was lower in rats that were defeated as adolescents than in control animals. The decrease in circadian amplitude correlates with the decrease in body weight (see Koolhaas et al., 1997) and also with a decrease in home cage locomotor activity (Meerlo et al., 1999) which is often interpreted as a failure to successfully cope with the stressor. In that sense, rats that were defeated as adolescents seemed to cope behaviorally and physiologically better with a similar exposure as adults to a residential male.

Experiment 1 showed that previously defeated rats were also attacked significantly later and received fewer attacks in total over both days. Observation of the behavior during the adult defeats clearly indicated that the animals defeated as adolescents were aware of the potential threat and were less explorative in the resident's cage. This may have caused the longer attack latency and the lower number of attacks. They apparently learned from their adolescent defeat experience how to optimize their behavior in this situation. This seems to be a clear illustration of "match" in the match–mismatch hypothesis (Ellis et al., 2011; Schmidt, 2011; Daskalakis et al., 2012) we referred to in the introduction. In the match–mismatch hypothesis it is claimed that the final consequence of childhood adversity depends on how well the early life environment matches the challenges in later life. In experiment 1 there is a clear match. Nonetheless, also previously defeated rats were attacked with a fierce intensity similar to that in the control animals. Behaviorally, the adolescent defeated rats and controls were similarly affected after the adult social defeat in their behavior in the elevated plus maze in which both groups of rats explored the open arms less than 10% of the total exploration time. Also the social interaction test resulted in a similar behavioral profile in both animal groups. These findings indicate that social defeat during adolescence alters play behavior but does not result in lasting increased vulnerability to later stressful challenges.

The interpretation of the results in experiment 2 basically leads to a similar conclusion although the "match" may be less clear in this experiment. The adolescent rats lived for a period of 1 week in a residential cage in which they were regularly confronted

with the resident. This is a slightly different procedure from being placed in the residential cage in the resident–intruder paradigm. The adolescent animals that were confronted repeatedly during 1 week with either a highly aggressive male WTG neighbor or a non-aggressive Wistar did not differ long-lastingly in the behavioral and physiological response to later challenges in life. Although the rats stressed as adolescents also tended to be attacked later and less frequently, this difference did not reach the level of significance like in experiment 1. This possibly is caused by the more evident match in situation in experiment 1 than in 2. The benefit of the adolescent experience may be less evident in experiment 2, but the data clearly show that the opposite, an increased behavioral and physiological response to adult stressful challenges, is not occurring in previously socially stressed individuals.

A few acute but minor changes in brain plasticity markers and behavior were observed but these changes were transient and no behavioral or physiological effects persisted into adulthood. As mentioned above, the social housing after the adolescent social defeat stress may have washed away the consequences of the social stress. It is also possible that adolescent rats are simply more resilient to the lasting consequences of social stress than adult animals. The behavioral observations of the adolescents during the social stress period indicated that behavior was significantly affected by both treatment and housing conditions. The defeated adolescents spent the majority of each 10-min interaction engaged in defeat-related behavior. Particularly the high freezing behavior and the avoidance of the adult male suggest that the adolescents housed next to an aggressive male rat did experience social stress. This conclusion is further supported by the decreased body weight gain in the defeated adolescents in comparison with the control-treated rats. Control adolescents did not show any of the defeat-related behavior nor did they show behavioral immobility or freezing. They spent significantly more time in social interactions as indicated by behavioral elements such as ‘‘play’’ and squeezing ‘‘under adult’’, which likely serves as an initiation of play. Single-housed control adolescents spent more time playing with the adult and less time exploring the cage than pair-housed control adolescents. Despite the aggressiveness of the WTG adult resident, even single-housed defeated adolescents would attempt to initiate social interaction by squeezing under the adult, whereas pair-housed defeated adolescents spent only little time initiating contact with the aggressive adult. The observation that adolescents attempt to interact with the adult even when this initiative inevitably leads to being attacked, demonstrates the strength of the social drive in 35–42-day-old adolescents.

Considering the effect of social stress on defensive burying in experiment 2, the timing of the stress during the adolescent developmental stage possibly plays an important role in the observed effects. Bingham et al. (2011) found that 1 week of daily social defeat between PND 28 and 34 increased burying behavior in single-housed adolescent rats, whereas the same treatment at PND 42–48 did not significantly affect burying behavior. In experiment 2 the adolescents were defeated between PND 35 and 42, i.e. between the two periods used by Bingham et al. (2011) who only found an effect in one age group. It is possible that the period between PND 35 and 42 represents a transition period during which adolescents move from one behavioral response to another and this could explain the lack of lasting behavioral changes in the present study. In another study applying social stress (Watt et al., 2009), it was shown that daily social defeat from PND 35 to 40 surprisingly caused a decreased anxiety in the elevated plus maze and an increased exploratory activity in the open field at PND 56. Only anxiety behavior in the context of the previous defeat stress increased. Also some mild changes in brain monoamine levels were detected at PND 63 in a small part of the brain areas analyzed. In that study adolescents were also socially housed after the defeat experiences (Watt et al., 2009).

A crucial element in experiment 2 was to see if the presence of a partner animal of similar age could buffer against the negative effects of prolonged social stress. The finding that social housing tended to facilitate instead of ameliorate the acute effects on body weight gain and changes in neuroplasticity markers like hippocampal cell proliferation and neurogenesis was rather unexpected. Previous studies in adult male rats exposed to social defeat followed by either isolation or social housing show a clear buffering effect of social housing on the stress-induced decrease on body weight gain (Koolhaas et al., 1997, 2011; Martinez et al., 1998; Ruis et al., 1999; de Jong et al., 2005). In adult rats, the social buffering effect is stronger when the experimental animal is housed with an unstressed partner than with a stressed partner (Kiyokawa et al., 2004), and even short time exposure to a stressed conspecific has been shown to alter the behavior in adolescent rats un-exposed to stress themselves (Jacobson-Pick et al., 2011). It is likely that the partner animals housed with defeated adolescents in this study are affected by the social defeat experience of the experimental animal and may have been stressed by the presence of the aggressive adult neighbor as well. Social housing between PND 22 and 46 has been shown to decrease body weight due to increased activity compared with single-housed adolescents (Zaias et al., 2008). The low body weight gain and the facilitation of changes in neuroplasticity markers in the pair-housed defeated adolescents in the present study is likely due to the combined effect of (i) defeat stress, (ii) continuous psychosocial stress caused by the neighboring aggressive adult, (iii) increased activity due to social housing and (iv) inability of the stressed partner animal to facilitate a buffering effect against the social defeat stress.

This study showed a remarkable ability in adolescents to recover from a socially adverse condition. Although this seems to be in contrast with some previous findings (Avital and Richter-Levin, 2005; Vidal et al., 2007; Watt et al., 2009; Buwalda et al., 2011), it does clearly indicate the relatively high resilience of adolescent rats to lastingly develop behavioral and neurobiological

stress pathologies. More studies examining and comparing acute and long-term effects of social and non-social stressors at different developmental stages under social as well as non-social housing conditions in the framework of the match–mismatch hypothesis are warranted before definite conclusions can be drawn concerning the sensitivity of specific age groups with regard to stress and its long-term consequences.

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